Oligonucleotide Inhibitors of bcl-xL

5   **Background of the Invention**

Throughout this application, various references are
referred to. Disclosures of these publications in
their entireties are hereby incorporated by reference
into this application to more fully describe the state
10   of the art to which this invention pertains.

Bcl-xL is an important anti-apoptotic protein that
belongs to the bcl-2 family. Bcl-xL is a critical
determinant of intimal lesion formation and thus is an
15   important contributor to the progression of vascular
disease. Pollman et al. (1998) Nature Medicine 4: 222-
227. In addition, bcl-xL has been implicated as a
causative factor in cancer.

20   The subject invention involves oligonucleotides that
reduce or eliminate the expression of bcl-xL.
Synthetic oligodeoxynucleotides have been utilized as
antisense inhibitors of mRNA translation *in vitro* and
in vivo. Beaucage, S., and Caruthers, M., (1981)
25   Tetrahedron Lett. 37:3557; Iverson, P. (1991) Anti-
Cancer Drug Des. 6:531; Ratajczak, et al. (1992) Proc.
Natl. Acad. Sci. U.S.A. 89:11823; Uhlmann, E., and
Peyman, A. (1990) Chem. Rev. 90:544-579. Antisense
oligonucleotides have found widespread application
30   because of their abilities to control and/or inhibit
gene expression in a selective manner in cellular
systems. Ghosh, S., et al. (1990) J. Biol. Chem.
265:2935-2940; Hemken, P., et al. (1992) J. Biol. Chem.
267:9948-0057; Lestinger, R., U.S. Patent No.
35   4,958,103, issued September 18, 1990; Shewmaker et al.,
U.S. Patent no. 5,107,065, issued April 21, 1992;
Tullis, U.S. Patent No., issued June 11, 1991; Zhao,
Q., et al. (1993) Antisense Research and Development
3:53-66. Thus, the subject invention represents a

precise and selective way of regulating bcl-xL expression both *in vivo* and *in vitro*.

Summary of the Invention

This invention provides an antisense oligonucleotide or analog thereof comprising 10 or more contiguous bases or base analogs from the sequence of bases of sequence A, B, C, D, E, F, G, H, I, J, K, L, or M of Figure 1.

This invention also provides the above-described antisense oligonucleotides, wherein the nucleotide sequence comprises nucleotide sequence A, A', B, C, C', D, E, E', F, G, G', H, H', I, I', J, K, K', L, L', M, or M' of Figures 2A and 2B.

This invention also provides the above-described antisense oligonucleotides, wherein the oligonucleotide is encapsulated in a liposome or nanoparticle.

This invention also provides the above-described antisense oligonucleotides, wherein the phosphate backbone comprises phosphorothioate bonds.

In addition, this invention provides a method of treating cancer, comprising introducing into a tumor cell an effective amount of the the above-described antisense oligonucleotide, thereby reducing the levels of bcl-xL protein produced and treating cancer.

This invention also provides the above-described methods, wherein the introducing comprises using amine and cationic delivery reagents.

This invention also provides the above-described methods, wherein the introducing comprises using porphyrin or lipofectin as a delivery agent.

This invention further provides the above-described pharmaceutical compositions, wherein the pharmaceutical composition comprises tetra meso-(4-

methylpyridyl)porphine or tetra meso-
(anilinium)porphine or a combination thereof.

Brief Description of the Figures

Figure 1
Oligonucleotide sequences complementary to bcl-X$_L$ mRNA.

Figures 2A and 2B
Oligonucleotide sequences and analogs thereof complementary to bcl-X$_L$ mRNA.

Figures 3A, 3B, 3C, and 3D
Some of the many possible phosphodiester moiety analogs.

Figure 4
An example of a sugar containing an aminoalkyloxy linker.

Figures 5A and 5B
Some of the many possible base moiety analogs.

Figure 6
Effect of 18-mer PS oligonucleotides on bcl-xL protein expression in LNCaP cells. The height of columns represents oligonucleotide inhibition of bcl-xL protein expression. Oligonucleotides were added to cells at a concentration of 1 μM in the presence of 3 μM of TAP for 4 h. The most active compound was T31062.

Figure 7
Western blot analysis of bcl-xL protein expression in prostate cancer cell lines after oligonucleotide treatment. The treatment of the cell with complex of oligonucleotides T31057, T31061, T31062 (1 μM) with TMP (2 μM) or TAP (3 μM) down-regulates bcl-xL protein expression, whereas oligonucleotides alone do not cause this effect.

Detailed Description of the Invention

The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

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C = Cytidine	A = Adenosine
T = Thymidine	G = Guanosine

Nucleic acid synthesizers are available to synthesize
10   oligonucleotides of any desired sequence. Certain oligonucleotide analogs may also be readily synthesized by modifying the reactants and reaction conditions. For example, phosphorothioate and methylphosphonate oligonucleotides may be synthesized using commercially
15   available automated oligonucleotide synthesizers.

An oligonucleotide's binding affinity to a complementary nucleic acid may be assessed by determining the melting tempature (T_M) of a
20   hybridization complex. The T_M is a measure of the temperature required to separate the nucleic acid strands of a hybridization complex. The T_M may be measured by using the hybridization complex's UV spectrum to assess the degree and strength of
25   hybridization. During hybridization, base stacking occurs which reduces the UV absorption of the nucleic acid. By monitoring UV absorption and the resulting increase in UV absorption that occurs during strand separation, one may assess the hybridization affinity
30   of a nucleic acid for its complement.

The structure and stability of hybridization complexes may be further assessed using NMR techniques known to those skilled in the art.

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A vast array of oligonucleotide analogs exist that achieve the same functionality as naturally occuring

oligonucleotides. There is an extensive literature
setting forth an almost limitless variety of
modifications that can be used to generate
oligonucleotide analogs. The phosphophate, sugar,
and/or base moieties may be modified and/or replaced by
the introduction/removal of chemical groups and/or
bonds. Many oligonucleotide analogs have superior
properties to those of naturally occurring
oligonucleotides. Such superior properties include,
but are not limited to, increased hybridization
affinity and/or resistance to degradation.

Phosphodiester Moiety Analogs

Numerous analogs to the naturally occurring
phosphodiester backbone have been used in
oligonucleotide design. Phosphorothioate,
phosphorodithioate, and methylphosphonate are readily
synthesized using known chemical methods. Because
novel nucleotide linkages can be synthesized manually
to form a dimer and the dimer later introduced into the
oligonucleotide via automated synthesis, the range of
potential backbone modifications is as broad as the
scope of synthetic chemistry. For example, the
oligonucleotide may be substituted or modified in its
internucleotide phosphate residue with a thioether,
carbamate, carbonate, acetamidate or carboxymethyl
ester. While all of the backbone modifications that
have been characterized are too broad to set forth,
Figures 3A, 3B, 3C, and 3D illustrate some of the many
backbone modifications that may be used in
oligonucleotide analogs.

Unlike the naturally occuring phosphodiester moieties,
many phosphodiester analogs have chiral centers. For
example, phosphorothioates, methylphosphonates,
phosphoramidates, and alkyl phosphotriesters all have
chiral centers. One skilled in the art would recognize

numerous other phosphodiester analogs that possess
chiral centers. Because of the importance of
stereochemistry in hybridization, the stereochemistry
of phosphodiester analogs can influence the
5   hybridization affinity of the oligonucleotide for its
target.

Most phosophodiester backbone analogs exhibit increased
resistance to nuclease degradation. In an embodiment,
10   phosphorothioates, methyl phosphonates,
phosphorimidates, and/or phosphotriesters are used to
achieve enhanced nuclease resistance. Increased
resistance to degradation may also be achieved by
capping the 5' and/or 3' end of the oligonucleotide.
15   In an embodiment, the 5' and/or 3' end capping of the
oligonucleotide is via a 5'-5' and/or 3'-3' terminal
inverted linkage.

Phosphorothioate oligodeoxynucleotides are relatively
20   nuclease resistant, water soluble analogs of
phosphodiester oligodeoxynucleotides. These molecules
are racemic, but still hybridize well to their RNA
targets. Stein, C., et al. (1991) Pharmac. Ther.
52:365-384.

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Phosphorothioate oligonucleotides may be stereo
regular, stereo non-regular or stereo random. A stereo
regular phosphorothioate oligonucleotide is a
phosphorothioate oligonucleotide in which all of the
30   phosphodiester linkages or phosphorothiodiester
linkages polarize light in the same direction. Each
phosphorous in each linkage may be either an Sp or Rp
diastereomer.

35   Sugar Moiety Analogs
Oligonucleotide analogs may be created by modifying
and/or replacing a sugar moiety.

The sugar moiety of the oligonucleotide may be modified by the addition of one or more substituents. For example, one or more of the sugar moieties may contain one or more of the following substituents: amino-alkylamino, araalkyl, heteroalkyl, heterocycloalkyl, aminoalkylamino, O, H, an alkyl, polyalkylamino, substituted silyl, F, Cl, Br, CN, CF_3, OCF_3, OCN, O-alkyl, S-alkyl, SOMe, SO_2Me, ONO_2, NH-alkyl, $OCH_2CH=CH_2$, OCH_2CCH, OCCHO, allyl, O-allyl, NO_2, N_3, and NH_2.

Modification of the 2' position of the ribose sugar has been shown in many instances to increase the oligonucleotide's resistance to degradation.

For example, the 2' position of the sugar may be modified to contain one of the following groups: H, OH, OCN, O-alkyl, F, CN, CF_3, allyl, O-allyl, OCF_3, S-alkyl, SOMe, SO_2Me, ONO_2, NO_2, N_3, NH_2, NH-alkyl, or $OCH=CH_2$, OCCH, wherein the alkyl may be straight, branched, saturated, or unsaturated.

In addition, the oligonucleotide may have one or more of its sugars modified and/or replaced so as to be a ribose or hexose (i.e. glucose, galactose).

Further, the oligonucleotide may have one or more α-anomeric sugars. The oligonucleotide may also have one or more L sugars.

The sugar may be modified to contain one or more linkers for attachment to other chemicals such as fluorescent labels. In an embodiment, the sugar is linked to one or more aminoalkyloxy linkers. An example of a sugar containing an aminoalkyloxy linker is shown in Figure 4. In another embodiment, the sugar contains one or more alkylamino linkers. Aminoalkyloxy and alkylamino linkers may be attached to biotin,

cholic acid, fluorescein, or other chemical moieties
through their amino group.

Base Moiety Analogs

In addition, the oligonucleotide may have one or more
of its nucleotide bases substituted or modified. In
addition to adenine, guanine, cytosine, thymine, and
uracil, other bases such as inosine, deoxyinosine,
hypoxanthine may be used. In addition, isoteric purine
2'deoxy-furanoside analogs, 2'-deoxynebularine or
2'deoxyxanthosine, or other purine or pyrimidine
analogs may also be used. By carefully selecting the
bases and base analogs, one may fine tune the
hybridization properties of the oligonucleotide. For
example, inosine may be used to reduce hybridization
specificity, while diaminopurines may be used to
increase hybridization specificity.

Adenine and guanine may be modified at positions N3,
N7, N9, C2, C4, C5, C6, or C8 and still maintain their
hydrogen bonding abilities. Cytosine, thymine and
uracil may be modified at positions N1, C2, C4, C5, or
C6 and still maintain their hydrogen bonding abilities.

Some base analogs have different hydrogen bonding
attributes than the naturally occurring bases. For
example, 2-amino-2'-dA forms three (3), instead of the
usual two (2), hydrogen bonds to thymine (T).

Examples of base analogs that have been shown to
increase duplex stability include, but are not limited
to, 5-fluoro-2'-dU, 5-bromo-2'-dU, 5-methyl-2'-dC, 5-
propynyl-2'-dC, 5-propynyl-2'-dU, 2-amino-2'-dA, 7-
deazaguanosine, 7-deazadenosine, and N2-
Imidazoylpropyl-2'-dG. For purposes of illustration,
several base analogs are shown in Figures 5A and 5B.

Pendant Groups

A "pendant group" may be linked to the oligonucleotide.
Pendant groups serve a variety of purposes which
include, but are not limited to, increasing cellular
uptake of the oligonucleotide, enhancing degradation of
the target nucleic acid, and increasing hybridization
affinity. Pendant groups can be linked to any portion
of the oligonucleotide but are commonly linked to the
end(s) of the oligonucleotide chain. Examples of
pendant groups include, but are not limited to:
acridine derivatives (i.e. 2-methoxy-6-chloro-9-
aminoacridine); cross-linkers such as psoralen
derivatives, azidophenacyl, proflavin, and
azidoproflavin; artificial endonucleases; metal
complexes such as EDTA-Fe(II), o-phenanthroline-Cu(I),
and porphyrin-Fe(II); alkylating moieties; nucleases
such as amino-1-hexanolstaphylococcal nuclease and
alkaline phosphatase; terminal transferases; abzymes;
cholesteryl moieties; lipophilic carriers; peptide
conjugates; long chain alcohols; phosphate esters;
amino; mercapto groups; radioactive markers;
nonradioactive markers such as dyes; and polylysine or
other polyamines.

In one example, the oligonucleotide comprises an
oligonucleotide conjugated to a carbohydrate, sulfated
carbohydrate, or gylcan. Conjugates may be regarded as
a way as to introduce a specificity into otherwise
unspecific DNA binding molecules by covalently linking
them to a selectively hybridizing oligonucleotide.

Cellular Uptake

To enhance cellular uptake, the oligonucleotide may be
administered in combination with a carrier or lipid.
For example, the oligonucleotide may be administered in
combination with a cationic lipid. Examples of
cationic lipids include, but are not limited to,

lipofectin, dotma, dope, and Dogs. The oligonucleotide may also be administered in combination with a cationic amine such as poly (L-lysine). Oligonucleotide uptake may also be increased by conjugating the oligonucleotide to chemical moieties such as transferrin and cholesteryls. In addition, oligonucleotides may be targeted to certain organelles by linking specific chemical groups to the oligonucleotide. For example, linking the oligonucleotide to a suitable array of mannose residues will target the oligonucleotide to the liver.

The cellular uptake and localization of oligonucleotides may be monitored by using labeled oligonucleotides. Methods of labeling include, but are not limited to, radioactive and fluorescent labeling. Fluorescently labeled oligonucleotides may be monitored using fluorescence microscopy and flow cytometry.

The efficient cellular uptake of oligonucleotides is well established. For example, when a 20 base sequence phosphorothioate (PS) oligonulceotide was injected into the abdomens of mice, either intraperitoneally (IP) or intravenously (IV). The highest concentrations of oligonucleotide accumulated in the kidney and liver, with only very small amounts being found in the brain. Chain-extended oligonucleotides were also observed. Argrawal, S., et al.(1988) Proc. Natl. Acad. Sci. U.S.A. 85:7079-7083. When the PS 27-oligonucleotide αrev was given IV or to rats, the initial $T_{1/2\alpha}$ (transit out of the plasma) was 23 min, while the $T_{1/2\beta}$ of total body clearance was 33.9 hours. The long β half-life of elimination demonstrates that dosing could be infrequent and still maintain effective, therapeutic tissue concentrations. Iverson, P. (1991) Anti-Cancer Drug Des. 6:531.

The efficacy of oligonucleotide therapy is also well established. For example, when a 24-base sequence PS oligonucleotide targeted to human c-myb mRNA was infused, through a miniosmotic pump, into scid mice bearing the human K562 chronic myeloid leukemia cell line, mean survival times of the mice treated with the antisense oligonucleotides were six- to eightfold longer than those of mice untreated or treated with the sense controls or treated with an oligonucleotide complementary to the c-kit proto-oncogene mRNA. Furthermore, significantly less tumor burden in the brain and ovary was observed histologically compared with the controls. After injecting IP 3'-PS- modified chimeric oligonucleotides that were complementary to the initiation codon region of the NF-κB mRNA (p65), a complete tumor involution was observed in 13 out of 13 antisense-treated mice. Untreated or sense-treated mice died by 12 weeks, where as the treated animals had no recurrence for at least 5 months. Ratajczak, et al. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:11823.

This invention provides an antisense oligonucleotide or analog thereof comprising 10 or more contiguous bases or base analogs from the sequence of bases of sequence A, B, C, D, E, F, G, H, I, J, K, L, or M of Figure 1.

This invention also provides an antisense oligonucleotide or analog thereof comprising a sequence having 90% of greater identity to sequence A, B, C, D, E, F, G, H, I, J, K, L, or M of Figure 1.

This invention also provides an antisense oligonucleotide or analog thereof comprising a sequence having 85% of greater identity to sequence A, B, C, D, E, F, G, H, I, J, K, L, or M of Figure 1.

This invention also provides an antisense

oligonucleotide or analog thereof comprising a sequence having 80% of greater identity to sequence A, B, C, D, E, F, G, H, I, J, K, L, or M of Figure 1.

5   This invention also provides an antisense oligonucleotide or analog thereof comprising a sequence having 75% of greater identity to sequence A, B, C, D, E, F, G, H, I, J, K, L, or M of Figure 1.

10   This invention also provides an antisense oligonucleotide or analog thereof comprising a sequence having 70% of greater identity to sequence A, B, C, D, E, F, G, H, I, J, K, L, or M of Figure 1.

15   This invention further provides an antisense oligonucleotide or analog thereof comprising nucleotide sequence A, B, C, D, E, F, G, H, I, J, K, L, or M of Figure 1.

20   This invention also provides the above-described antisense oligonucleotides, wherein the nucleotide sequence comprises nucleotide sequence A, A', B, C, C', D, E, E', F, G, G', H, H', I, I', J, K, K', L, L', M, or M' of Figures 2A and 2B.

25   This invention further provides the above-described antisense oligonucleotides, wherein the oligonucleotide is conjugated to a peptide.

30   This invention also provides the above-described antisense oligonucleotides, wherein the oligonucleotide is encapsulated in a liposome or nanoparticle.

This invention also provides the above-described
35   antisense oligonucleotides, wherein the phosphate backbone comprises phosphorothioate bonds.

In addition, this invention provides the above-described antisense oligonucleotides, wherein the backbone is bonded to one or more lipid substituents.

5   This invention also provides the above-described antisense oligonucleotides, wherein one or more of the oligonucleotides's sugars contain an -OMe group at their 2' positions.

10   This invention further provides the above-described antisense oligonucleotides, wherein the phosphate backbone consists essentially of phosphorothioate bonds.

15   This invention also provides the above-described antisense oligonucleotides, wherein the phosphorothioate is stereo regular.

This invention also provides the above-described
20   antisense oligonucleotides, wherein the oligonucleotide is linked to an intercalating agent, a cross-linker, an endonuclease, a lipophilic carrier, an alkylating agent, a coordination complex, or a peptide conjugate, or a combination thereof

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In addition, this invention provides the above-described antisense oligonucleotides, wherein the oligonucleotide is modified to reduce its ionic charge or increase its hydrophobicity.

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In an embodiment, an alkyl group is attached to increase the hydrophobicity of the oligonucleotide. In another embodiment, one or more oxygen atoms are replaced by sulphur atoms to decrease the
35   hydrophobicity of the oligonucleotide.

This invention also provides the above-described

antisense oligonucleotides, wherein the oligonucleotide
comprises one or more short chain alkyl structures that
replace some of the oligonucleotide's phosphodiester
bonds.

5   This invention further provides the above-described
antisense oligonucleotides, wherein the oligonucleotide
is linked to one or more cholesteryl moieties.

10   This invention also provides the above-described
antisense oligonucleotides, wherein the
oligonucleotides comprises one or more bases with a C-5
propynyl pyrimidine modification.

15   In addition, this invention provides a method of
treating cancer, comprising introducing into a tumor
cell an effective amount of the the above-described
antisense oligonucleotide, thereby reducing the levels
of bcl-xL protein produced and treating cancer. In an
20   embodiment, the cancer being treated is epithelial
cancer.

The actual effective amount will be based upon the size
of the oligonucleotide, the biodegradability of the
25   oligonucleotide, the bioactivity of the oligonucleotide
and the bioavailability of the oligonucleotide. If the
oligonucleotide does not degrade quickly, is
bioavailable and highly active, a smaller amount will
be required to be effective. The effective amount will
30   be known to one of skill in the art; it will also be
dependent upon the form of the oligonucleotide, the
length of the oligonucleotide and the bioactivity of
the polypeptide. One of skill in the art could
routinely perform empirical activity tests to determine
35   the bioactivity in bioassays and thus determine the
effective amount.

This invention also provides the above-described methods, wherein the effective amount is between 0.1 μM and 4 μM.

5   In addition, this invention provides the above-described methods, wherein the effective amount is between 0.4 μM and 1 μM.

This invention further provides the above-described
10   methods, wherein the cancer is epithelial cancer.

This invention also provides the above-described methods, wherein the epithelial cancer is prostate cancer.

15   This invention also provides the above-described methods, wherein the epithelial cancer is lung cancer.

This invention also provides the above-described
20   described methods, wherein the epithelial cancer is bladder cancer.

This invention also provides the above-described methods, wherein the introducing comprises using a
25   lipid as a delivery agent.

This invention further provides the above-described methods, wherein the introducing comprises using porphyrin or lipofectin as a delivery agent.
30
This invention also provides the above-described methods, wherein the effective amount is between 0.1 μM and 4 μM.

35   In addition, this invention provides the above-described methods, wherein the effective amount is between 0.4 μM and 1 μM.

This invention also provides a method of promoting the
regression of vascular lesions, comprising introducing
into a vascular cell an amount of the the above-
described antisense oligonucleotides effective to
reduce the levels of bcl-xL protein produced, thereby
promoting the regression of vascular lesions.

This invention further provides the above-described
methods, wherein the introducing comprises using a
lipid as a delivery agent.

This invention also provides the above-described
methods, wherein the introducing comprises using
porphyrin or lipofectin as a delivery agent.

This invention also provides the above-described
methods, wherein the effective amount is between 0.1 μM
and 4 μM.

In addition, this invention provides the above-
described methods, wherein the effective amount is
between 0.4 μM and 1 μM.

This invention also provides a pharmaceutical
composition comprising an effective amount of any of
the above-described antisense oligonucleotides or
analogs thereof and a pharmaceutically acceptable
carrier.

Pharmaceutically acceptable carriers are well known to
those skilled in the art and include, but are not
limited to phosphate buffer and saline. Additionally,
such pharmaceutically acceptable carriers may be
aqueous or non-aqueous solutions, suspensions, and
emulsions. Examples of non-aqueous solvents are
propylene glycol, polyethylene glycol, vegetable oils
such as olive oil, and injectable organic esters such

as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention further provides the above-described pharmaceutical compositions, wherein the effective amount is between 0.1 μM and 4 μM.

In addition, this invention provides the above-described pharmaceutical compositions, wherein the effective amount is between 0.4 μM and 1 μM.

This invention also provides the above-described pharmaceutical compositions, wherein the oligonucleotide is encapsulated in a liposome or nanoparticle.

This invention further provides the above-described pharmaceutical compositions, wherein the pharmaceutical composition comprises tetra meso-(4-methylpyridyl)porphine or tetra meso-(anilinium)porphine or a combination thereof.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully

in the claims which follow thereafter.

Forty 18- and 20-mer mixed phosphate/phosphorothioate backbone oligonucleotides with or without C-5-propyne pyrimidine modifications chosen by "walking" along the bcl-xL mRNA have been evaluated as to their ability to decrease bcl-xL protein expression in human prostate cancer cell lines (LNCaP, DU145 and PC3). The cell lines were obtained from the ATTC (Rockville, MD). The cells were grown in RPMI 1640 media supplemented with 10% FBS in 5% CO_2 atmosphere and routinely passaged when 90-95% confluent. All oligonucleotides were at least 95% full-length material when analysed by a reverse-phase HPLC.

Two novel agents for the intracellular delivery of oligonucleotides were used: tetra meso-(4-methylpyridyl)porphine (TMP) and tetra meso-(trimethylammonium)porphine (TAP). TMP and TAP both are cations which form 2:1 and 3:1 complexes with oligonucleotides. Human prostate carcinoma cells were plated in 6-well tissue culture dishes and next day (when 80-90% confluent) were washed once with OPTI-MEM and treated with prepared complex of oligonucleotide (1 μM) plus TMP (2 μM) or TAP (3 μM) for 4 h at 37°C. The cells were then washed once with RPMI-10% FBS and were allowed to recover in RPMI-10% FBS for another 20 h. At this time cells were washed twice with PBS and then protein and/or mRNA were extracted and analysed.

Western blot analysis demonstrated the effective down-regulation of bcl-xL protein with five of the most active oligonucleotides (T31057, T31061, T31062, T31065, T31067). For the western blot analyses approximately 10^6 cells were lysed for 30 min on ice in 100 μl of RIPA buffer (50 mM Tris-HCl, pH 8.0 - 150 mM NaCl - 0.1% SDS - 1% NP40 - 0.5% sodium deoxycholate).

Aliquots of cell extracts containing 20 μg of protein were electrophoresed in 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Corp., Bedford, MA). Filters were blocked and stained with an 1:200 dilution of rabbit anti-bcl-x polyclonal antibody (Santa Cruz Biotechnology Inc., Santa-Cruz, CA). After washing, filters were incubated in a 1:10,000 dilution of peroxidase-conjugated anti-rabbit secondary antibody (Amersham Life Sciences, Arlington Heights, IL). ECL was performed using the procedure recommended by the manufacturer and the filters were exposed to X-ray film.

Control oligonucleotides did not affect expression of bcl-xL and cell viability. The most active oligonucleotides are complementary to regions on the 3'-end of the bcl-xL mRNA. Moreover, C-5 propyne pyrimidine modified oligonucleotides seem to be more effective than pyrimidine unmodified.

These five active oligonucleotides (T31057, T31061, T31062, T31065, T31067) alone did not induce apoptosis in DU145 prostate cancer cell lines, as is shown with DAPI-staining. To determine the amount of apoptotic nuclei, cells were plated in 4-chamber tissue culture slides (Nunc, Inc., Naperville, IL) and after the experiment, were washed with PBS, fixed with 90% ethanol/ 5% acetic acid, and after 2 rinses with PBS, stained with a 1.5 mg/ml solution of DAPI in PBS. The slides were washed twice with PBS, mounted and photographed using a Nikon phase-fluorescence microscope.

In an attempt to approximate clinical drug resistance in prostate cancer a commonly used prostate cancer cell line, LNCaP, was genetically manipulated so that these cells would overexpress the human bcl-xL protein. This

cell line is a popular model for the study of prostate cancer, because it retains some of the most prominent differentiated features of the human prostate cell. LNCaP cells also have proven to be growth responsive to androgen steroids in vitro. This bcl-xL overexpressing cell line provides a useful in vitro model for the subsequent study of drug resistant adenocarcinoma of the prostate gland.

The human prostate cancer cell line LNCaP was received from ATTC (Rockville, MD). LNCaP cells were propagated as monolayer culture in RPMI 1640 supplemented with 10% FBS, in 5% CO_2 atmosphere. Confluent cultures were routinely passaged approximately weekly, with RPMI 1640, containing 5 mM EDTA.

LNCaP cells transfected with the neomycin-selectable pSFFV/bcl-xL plasmid or with a control, neomycin-resistant expression vector pSFFV. Aliquots containing 10 μg of plasmid and 5 μg of lipofectin reagent (Life technologies, Inc., Gaithersburg, MD) in serum-free OPTI-MEM (Life Technologies, Inc.) were added to cultured cells. The transfection media was replaced 4 h later with RPMI-10% FBS medium. Individual colonies were selected from these plates after approximately 3-4 weeks of routine maintenance in RPMI-10% FBS containing 0.6 mg/ml G418 sulfate (Geneticin, Life Technologies, Inc.). The bcl-xL-transformed LNCaP cells were cultured in RPMI-10% FBS, supplemented with 0.3 mg/ml G418 sulfate in 5% CO_2 atmosphere.

Two clones of LNCaP cells overexpressing bcl-xL protein (1072-4 and 1072-5) have been obtained after transfection of wild type LNCaP cells with the plasmid vector pSFFV/bcl-xL and lipofectin. Also a mock transfectant clone of LNCaP cells carrying neo®

resistance gene (1072-3) was used for the control experiments. Clone 1072-4 demonstrates 10-fold overexpression, and clone 1072-5 - 4-fold overexpression of bcl-xL protein. Western blot analysis for bcl-xL protein was performed as described above. Results for bcl-xL protein expression were confirmed by Northern blot analysis for bcl-xL mRNA expression, demonstrating significant elevation of this mRNA in bcl-xL transformed cell lines. For the Northern blot analysis, the total RNA was isolated from the cells using TRIZOL reagent (GIBCO BRL), and 20 μg aliquotes were separated in RNA-formaldehyde gel, blotted onto nylon membranes (Schleicher & Schull), UV-linked and prehybridized for two hours at 42 °C in the standard hybridization solution. Then the blot was hybridized overnight with the PCR-amplified fragment of human bcl-xL cDNA at 42 °C. Bcl-xL coding fragment was amplified from pSFFV/bcl-xL plasmid using bcl-x specific primers. The primer sequences were: bcl-x-upstream, 5'-ATGTCTCAGAGCAACCGGGA-3'; and bcl-x-downstream, 5'-TCATTTCCGACTGAAGAGTG-3'. Twenty five cycles of amplification were performed in DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT) at 94 °C (30 sec), 55 °C (30 sec), and 72 °C (30 sec). The PCR products were analysed on a 1.2% agarose gel. The resultant fragment was labeled by random primer method to the specific activity 10^7 cpm/ng of the probe and used for the hybridization. After washings blots were autoradiographed for 24h at -80 °C. Blots were stripped of radioactivity and reprobed with a ^{32}P-labeled G3PDH probe to confirm the equal loading.

Overexpression of bcl-xL protein does not affect the relative growth rate of LNCaP cells in culture medium containing 10% FBS as determined by MTT assay. For this assay LNCaP cells were seeded in 24-well tissue culture plates. Next day after the experiment the old media was

removed, the cells were washed once with PBS, and RPMI
containing 0.5 mg/ml MTT (Sigma Co, St. Louis, MO) was
added. The cells were incubated at 37°C for 4 h, then
solubilization solution (0.04N HCl in isopropyl
5   alcohol) was added and optical density was measured at
540 nm.

Clonal derivatives of bcl-xL transfected LNCaP cells
that were overexpressing high amounts of bcl-xL protein
10   were resistant to taxol (up to 100 nM) and mitoxantrone
(up to 4 μM) treatment for 24 h. These drugs have been
shown to induce apoptosis in parental LNCaP cells or
neomycin-expressing control-transfected LNCaP cells.
This finding strongly supports the hypothesis that
15   bcl-xL gene product is a factor in the development of
chemotherapy resistance in hormone-refractory prostate
cancer.

Antisense oligonucleotides provide an efficient means
20   for bcl-xL protein elimination in prostate cancer cell
lines. When antisense oligonucleotides were applied to
the bcl-xL overexpressor, the down-regulation of this
anti-apoptotic protein was not apparant. A possible way
to down-regulate bcl-xL protein in such case could be
25   using antisense oligonucleotide therapy in combination
with chemotherapeutic drugs.

It has been shown recently (Am J Pathol 1996,
148:1567-1576) that expression of several
30   anti-apoptotic members of the bcl-2 family, including
bcl-2 and bcl-xL proteins increases during progression
of prostate cancers. Our transfected clones
overexpressing bcl-xL can mimic the clinical model of
adenocarcinoma of the prostate gland. Demonstration of
35   the mechanism of action of different drugs and their
combinations with antisense oligonucleotides in these
transfected LNCaP cells could help form the basis for a

better understanding of prostate cancer.

Forty modified oligonucleotides complementary to the bcl-xL mRNA have been evaluated as to their ability to decrease bcl-xL protein expression in human prostate cancer cell lines (LNCaP, DU145, and PC3).

Two novel agents for the intracellular delivery of oligonucleotides were used: tetra meso(4-methylpyridyl)porphine (TMP) and tetra meso(trimethylammonium)porphine (TAP).

Western blot analysis demonstrated effective down-regulation of bcl-xL protein with five of the most active oligonucleotides (T31057, T31061, T31062, T31065, T31067).

These active oligonucleotides alone did not induce apoptosis in DU145 cells.

Two clones of LNCaP cells overexpressing bcl-xL protein and clone carrying neo resistance gene only have been obtained after transfection of wild type LNCaP cells with the plasmid vectors pSFFV/bcl-xL and pSFFV.

Overexpression of bcl-xL protein and mRNA has been shown by western and northern blot analyses.

Overexpression of bcl-xL protein does not affect the relative growth rate of LNCaP cells in culture medium containing 10% FBS.

Bcl-xL overexpressing clones of LNCaP cell lines demonstrated high resistance to taxol (100 nM) and mitoxantrone (4 μM) treatment.

Active antisense oligonucleotides do not down-regulate

bcl-xL protein in the LNCaP clones overexpressing
bcl-xL.